





# Size-selective mortality induces evolutionary changes in group risk-taking behaviour and the circadian system in a fish

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## Abstract

1. Intensive and trait-selective mortality of fish and wildlife can cause evolutionary changes in a range of life-history and behavioural traits. These changes might in turn alter the circadian system due to co-evolutionary mechanisms or correlated selection responses both at behavioural and molecular levels, with knock-on effects on daily physiological processes and behavioural outputs.
2. We examined the evolutionary impact of size-selective mortality on group risk-taking behaviour and the circadian system in a model fish species. We exposed zebrafish *Danio rerio* to either large or small size-selective harvesting relative to a control over five generations, followed by eight generations during which harvesting was halted to remove maternal effects.
3. Size-selective mortality affected fine-scale timing of behaviours. In particular, small size-selective mortality, typical of specialized fisheries and gape-limited predators targeting smaller size classes, increased group risk-taking behaviour during feeding and after simulated predator attacks. Moreover, small size-selective mortality increased early peaks of daily activity as well as extended self-feeding daily activity to the photophase compared to controls. By contrast large size-selective mortality, typical of most wild capture fisheries, only showed an almost significant effect of decreasing group risk-taking behaviour during the habituation phase and no clear changes in fine-scale timing of daily behavioural rhythms compared to controls.
4. We also found changes in the molecular circadian core clockwork in response to both size-selective mortality treatments. These changes disappeared in the clock output pathway because both size-selected lines showed similar transcription profiles. This switch downstream to the molecular circadian core clockwork also resulted in similar overall behavioural rhythms (diurnal swimming and self-feeding in the last hours of darkness) independent of the underlying molecular clock.
5. To conclude, our experimental harvest left an asymmetrical evolutionary legacy in group risk-taking behaviour and in fine-scale daily behavioural rhythms. Yet, the overall timing of activity showed evolutionary resistance probably maintained by

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a molecular switch. Our experimental findings suggest that size-selective mortality can have consequences for behaviour and physiological processes.

#### KEYWORDS

activity, boldness, circadian clock, circadian switch, feeding behaviour, fisheries-induced evolution, gene expression, life history

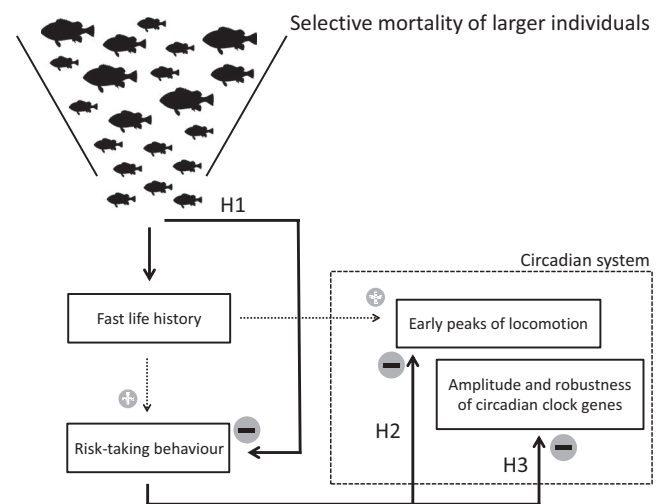
## 1 | INTRODUCTION

Harvesting is among the five major forms of human-induced environmental change (Díaz et al., 2019). Harvesting differs from many other forms of natural predation by primarily targeting adult individuals—a size class that typically experiences limited natural mortality (Darimont et al., 2015). Intensive and trait-selective harvesting thus can shift the fitness landscape and foster evolutionary adaptations at a rate and speed that is rarely experienced in the evolutionary history of many animal populations (Allendorf & Hard, 2009; Jørgensen et al., 2007; Palumbi, 2001; Sih et al., 2011).

Empirical and modelling studies on the topic of fisheries-induced evolution have suggested intensive fishing fosters evolution of a fast life history that is characterized by elevated reproductive investment, reduced age and size at maturation and reduced post maturation growth and longevity (Heino et al., 2015; Jørgensen et al., 2007). Fisheries-induced evolution might also affect behavioural and physiological traits due to direct selection, co-evolutionary mechanisms and correlated selection responses (Hollins et al., 2018; Uusi-Heikkilä et al., 2008). However, behavioural and physiological adaptations to fishing are much less studied compared to adaptive changes in life-history traits (Heino et al., 2015).

The proximate mechanisms governing fisheries-induced evolution of behaviour, as well as other traits, are difficult to disentangle in the wild because phenotypic changes observed in time series (e.g. changes in average age at maturation) can be masked by phenotypic plasticity, i.e. non-genetic changes in trait expression (Heino et al., 2015). One means to single out the cause-and-effect of fishing is conducting selection experiments with model species in the laboratory (Conover & Baumann, 2009; Diaz Pauli & Heino, 2014). Several laboratories have investigated the evolutionary effects of size-selective harvesting on behaviour by means of selection experiments with different model fish species (e.g. Diaz Pauli et al., 2019; Philipp et al., 2009; Uusi-Heikkilä et al., 2015; Walsh et al., 2006). This line of research has largely focused on evolutionary changes of individual behavioural traits such as boldness. However, a focus on group behaviour is crucial to fully understand the evolutionary changes in response to fishing or other forms of natural mortality because collective behaviours such as shoaling might be key drivers of the capture process (e.g. Parrish, 1999; Tenningen et al., 2016) and could also have adaptive value to reduce exposure to natural predators (Pitcher, 1986; Sumpter, 2010). More work is needed to understand whether and to what degree intensive size-selective mortality can affect individual and group behavioural traits in fish and other species (Andersen et al., 2018; Arlinghaus et al., 2017; Diaz Pauli & Sih, 2017).

Size-selective harvesting can affect behavioural traits through at least two mechanisms (Figure 1). First, following the pace-of-life syndrome (Réale et al., 2010), behavioural and life-history traits are often correlated along a fast-to-slow continuum. Fast life-history traits—those trait combinations favoured under intensive and large size-selective harvesting pressure (e.g. Andersen et al., 2018; Jørgensen & Holt, 2013)—are expected to covary and co-evolve with an increase of risk-taking behaviour (Réale et al., 2010; Wolf



**FIGURE 1** The conceptual framework of the putative mechanisms driving evolution of risk-taking behaviour and circadian system in response to size-selective mortality. The figure showed large size-selective mortality typical of many fisheries and specialized predators targeting larger size classes. The same conceptual framework with opposing outcomes is valid for small size-selective mortality typical of specialized fisheries and most gape-limited predators. In particular, large size-selective harvesting triggers the evolution of a fast life history, which in turn can co-evolve with an increase of both risk-taking behaviour and early peaks of locomotor activity (small dotted arrows). However, a plausible scenario is that harvesting disrupts the correlation of life history with other behavioural traits. Therefore, a further possibility is that large-size selective harvesting triggers the evolution of a decrease risk-taking behaviour through a correlated selection response with size-at-harvest (bold arrow and first hypothesis tested here; H1). Consequently, a decrease of risk-taking behaviour may affect the circadian system by triggering a decrease of early locomotor activity rhythms (bold arrow and second hypothesis; H2), and reducing amplitude and robustness of circadian clock gene expression (bold arrow and third hypothesis; H3). For more details, see the main text (Fish pic source: <https://pixy.org/4660397/>)

et al., 2007; Figure 1). The argument is that acquiring the resources needed to maintain a fast life history (i.e. rapid juvenile growth or high reproductive investment) favours risky behaviours, such as feeding, in the presence of predators. Under such scenario, large size-selective harvesting is expected to evolutionarily increase risk-taking behaviour (Figure 1). Yet, recent refinements of the pace-of-life syndrome hypothesis suggest that specific ecological contexts (e.g. variation in natural mortality risk) could systematically break apart the genetic correlation of life-history and behavioural traits (Dammhahn et al., 2018; Montiglio et al., 2018). Indeed, observational (Dhellemmes et al., 2020; Polverino et al., 2018) and theoretical (Andersen et al., 2018; Claireaux et al., 2018) studies in fish have suggested that the covariance of life-history and risk-taking behavioural traits may change in response to mortality-based selection pressures. It is thus equally plausible that intensive and large size-selective harvest may foster the evolution of both a fast life history and a decrease of risk-taking behaviour, in particular when size-selection is strongly directed to very large and mature individuals (Andersen et al., 2018).

Second, fish behavioural traits can evolve in response to correlated selection responses linked to size-selective mortality due to a systematic positive correlation of specific behaviours, growth rate and size-at-age (Biro & Post, 2008; Biro & Sampson, 2015; Klefoth et al., 2017). Specifically, behavioural traits related to resource acquisition, such as risk-taking behaviour during foraging (defined as boldness), increase food intake and thereby elevate growth rate and affect size-at-age (Enberg et al., 2012). In scenarios where the larger individuals have a higher risk of mortality due to fishing or to specialized natural predators, selective removal of these larger, faster-growing individuals might also indirectly favour a decrease of risk-taking behaviour (Biro & Post, 2008). Consequently, selective harvesting of large fish might evolutionarily favour a decrease of risk-taking behaviour (Figure 1).

Evolutionary changes in behavioural traits can in turn affect the circadian system (Figure 1). Recent studies have reported a relationship between copying style (or animal personality), particularly risk-taking behaviour and the circadian system in zebrafish *Danio rerio* (Rey et al., 2013; Tudorache et al., 2018). The circadian system is widely conserved phylogenetically (Panda et al., 2002) and controls daily rhythms of physiology and behaviour (Dunlap et al., 2004). Thereby, circadian rhythms fulfill an adaptive function helping the organism to anticipate and maintain synchronization to external 24-hr environmental cycles (Cowan et al., 2017; Idda et al., 2012; Kronfeld-Schor & Dayan, 2003). Functional circadian clocks are essential for precise timing of activity, thereby helping the organism to avoid or minimize the risk of predation while maximizing foraging (DeCoursey et al., 2000).

Evolutionary changes of the circadian system in exploited populations may also occur due to the interplay between the evolutionary responses to life history and behaviour (Figure 1). Experimental evolution in *Drosophila melanogaster* indicated that selection for slower-running circadian clocks could result in slower rates of developmental processes, which in turn can delay development time

(vice versa for faster-running circadian clocks; Nikhil & Sharma, 2017 and references therein). Moreover, the clock gene *clock* (i.e. a gene that is part of the core transcription-translation feedback loop of the molecular circadian clockwork in fish; Vatine et al., 2011) is mainly mapped in regions related to life-history traits in salmonids (Leder et al., 2006; Paibomesai et al., 2010). This suggests that life history changes induced by harvesting could co-evolve with the circadian clock (e.g. Leder et al., 2006; Nikhil & Sharma, 2017). Moreover, proactive (i.e. more risk taking) zebrafish have shown more robust diurnal rhythms at molecular, endocrine and behavioural level than reactive ones (Tudorache et al., 2018). Therefore, harvesting-induced evolution of risk-taking behaviour can also be expected to affect the circadian system both at behavioural and molecular levels (Figure 1). Studying both levels is relevant because different molecular processes can underlie similar phenotypic outcomes, as recently demonstrated in a harvesting-induced evolution experiment using Atlantic silversides, *Menidia menida* (Therkildsen et al., 2019).

We take advantage of a long-term harvesting experimental system based on zebrafish (Uusi-Heikkilä et al., 2015). The zebrafish selection lines were exposed to strong directional harvest selection (a 75% per-generation harvest rate) acting on either large (large-harvested line; a common scenario in many fisheries world-wide and in presence of predators where large individuals are selectively preyed upon) or small (small-harvested line; a possible scenarios in specific fisheries or in the presence of gape-limited predators that preferentially feed on the smaller size classes) fish, relative to a control line harvested randomly with respect to body size (Uusi-Heikkilä et al., 2015). Previous research has shown that the large-harvested line evolved a faster life history characterized by smaller adult length and weight, higher relative fecundity and elevated senescence compared to controls (Uusi-Heikkilä et al., 2015; see Figure S1). By contrast, the small-harvested line showed a slower life history compared to controls, characterized by reduced reproductive investment and no change in adult length compared to the control line (Uusi-Heikkilä et al., 2015). Other previously documented changes include evidence for genetic and genomic changes, i.e. the differences among the selection lines were indicative of evolutionary and not just plastic adaptations (Uusi-Heikkilä et al., 2015, 2017). Size-selection occurred during the first five generations, but the size-selected lines maintained their key life history adaptations after harvesting halted for up to eight generations (Sbragaglia, Gliese, et al., 2019; Uusi-Heikkilä et al., 2015), indicating that harvesting-induced evolution of life-history traits did not recover to pre-harvesting levels (Figure S1). The fixation of the harvesting-induced changes in life history after harvesting halted is a precondition to allow a comparison among the selection lines in terms of harvesting-induced evolution of behaviour and the circadian system. Addressing this question was the aim of the present research (Figure 1).

We predicted (H1 in Figure 1) that evolution of group risk-taking behaviour is governed by a resource acquisition mechanism in a way that faster growth rate is correlated with an increase of group risk-taking behaviour (Enberg et al., 2012). Therefore, the selective removal of the larger, faster-growing individuals is predicted to result in the evolution of decreased group risk-taking behaviour in the large-harvested

line, and vice versa for the small-harvested line. Second, we predicted that a decrease of group risk-taking behaviour in the large-harvested line triggers changes in the circadian system both at behavioural and molecular levels (Rey et al., 2013; Tudorache et al., 2018). If this is true, we expected the absence of early peaks of locomotor activity (H2 in Figure 1), because a decrease of risk-taking behaviour has been associated with the absence of clear peaks of activity in zebrafish (Tudorache et al., 2018), and *vice versa* for the small-harvested line. Third, at the molecular level, we expected that the large-harvested line lost circadian rhythmicity of clock genes (H3 in Figure 1), because a decrease of risk-taking behaviour has been associated with a loss of robustness and amplitude in clock genes expression (here quantified as circadian rhythmicity; Tudorache et al., 2018), and *vice versa* for the small-harvested line. Following well-established knowledge of the functioning of the zebrafish circadian system (e.g. Idda et al., 2012; Vatine et al., 2011), we focused our investigation on carefully selected genes related to fundamental properties of the circadian system functioning, such as the core transcriptional-translational feedback loop (Dunlap et al., 2004; Vatine et al., 2011), light-inducible genes (genes directly activated by light that can interfere with the functioning of the core feedback loop; Tamai et al., 2007; Vatine et al., 2011), circadian clock output (genes related to mechanisms downstream the core feedback loop) and clock-controlled genes related to energy balance.

## 2 | MATERIALS AND METHODS

### 2.1 | Selection lines

Our experimental system consisted of wild-collected zebrafish from West Bengal in India, sampled with a range of fishing gears (seine, cast nets and dip nets). The parental wild-collected population was experimentally harvested as explained in detail elsewhere (Uusi-Heikkilä et al., 2015). Each selection line was replicated twice for a total of six selection lines, similar to a landmark study about the outcomes of experimental size-selective harvesting in Atlantic silversides (Conover & Munch, 2002; Therkildsen et al., 2019). Zebrafish were exposed to size-selection only during the first five generations and then harvesting halted for several further generations (Uusi-Heikkilä et al., 2015) to remove maternal effects and study evolutionary outcomes in a common-garden setting. Age at harvest varied from generation to generation associated with potential changes in age at 50% maturation of the random line, and each selected parental fish was only able to spawn once. Therefore, the experimental design did not allow the estimation of selection responses typical of those expected in overlapping generations with respect to age at maturation.

Zebrafish selection lines for the present experiment where from  $F_{13}$ , eight generations after harvesting halted. The selection lines maintained key life-history adaptations. The large-harvested line showed a fast life-history (e.g., elevated reproductive investment and reduced post maturation growth) and the small-harvested line signs of a slow fast-history (e.g., reduced reproductive investment; Figure S1; Uusi-Heikkilä et al., 2015). Fish were reared in groups under ad libitum

feeding and maintained under the following conditions: water temperature at  $26 \pm 0.5^\circ\text{C}$ ; photoperiod at 12:12 hr Light-Darkness (LD) cycle (lights on/off at 07:00 and 19:00 respectively); fed three times per day with dry food (TetraMin, Tetra, Germany) mainly in the first part of the light hours (at 09:00; 11:00 and 13:00). Fish were reared and manipulated following the guidelines of the European Union (2010/63/EU) and the Spanish legislation (RD 53/2013 and Law 32/2007). The experimental protocols were approved by the Spanish National Committee and the Committee of the University of Murcia on Ethics and Animal Welfare (reference number A13191003).

Behavioural traits and circadian rhythms are sensitive to social modulation (Bloch et al., 2013; Castillo-Ruiz et al., 2012; Jolles et al., 2017), and previous results showed that the selection lines behaved differently when tested in isolation or in groups (Sbragaglia, Alós, et al., 2019). Indeed, being in a group has important consequences for foraging behaviour in zebrafish (Harpaz & Schneidman, 2019; Pitcher, 1986), and social isolation can create stress-related behaviours (Shams et al., 2017). Therefore, knowing that zebrafish is a social species (Spence et al., 2008), and the size-selective harvesting treatments occurred in a social environment, we studied zebrafish behaviour at the group level for testing our hypotheses.

### 2.2 | Group risk-taking behaviour experiments

We first characterized the diving behaviour of zebrafish to test whether the selective removal of the larger individuals triggered a decrease of group risk-taking behaviour (*vice versa* for the selective removal of smaller individuals; H1 in Figure 1). Groups of eight juveniles (30 days post fertilization; dpf) were stocked into 3-litre rearing boxes. The boxes ( $N = 36$ ) were housed on the shelves of the same zebrafish holding system with a randomized order (six replicates for each line; 12 replicates per treatment). Throughout the experiment zebrafish were fed ad libitum with dry food (TetraMin, Tetra, Germany) and maintained in the same conditions reported above. Measurements of diving behaviour were assessed with adult individuals at 230 and 240 dpf to estimate consistent inter-group differences as a measure of collective personality. To that end, the groups of eight zebrafish were moved from the 3-L rearing box in a new experimental tank (width  $\times$  length  $\times$  height =  $10 \times 30 \times 25$  cm) with 22 cm of water (Figure S2). The experimental tank was placed on a table behind a white curtain. On the side of the experimental tank (at about 50 cm), we placed a webcam (Figure S2; Video S1) and measured the cumulative time any individual of the shoals spent at the surface (top 7 cm of the tank; Figure S2) during an experimental assay with duration of 6 min and 30 s composed of: an acclimation period (3 min), a feeding period (30 s), the approach of a simulated predator (5 s) and the rest of time after the predator approach (3 min; Figure S2; Video S1). The time spent at the surface is a well-established behavioural test used in zebrafish to measure anxiety-like behaviour (Egan et al., 2009; Kalueff, 2017; Levin et al., 2007). Zebrafish show a typical diving response moving towards the bottom of the tank as soon as they are introduced in a novel tank, followed

by a slow exploration of the surface (Kalueff, 2017). The surface of the water is a risky environment for zebrafish (Spence et al., 2008), and previous experimental results showed that stimuli mimicking an approaching predator from the top of the tank induced a robust increase of time spent at the bottom of the tank (Luca & Gerlai, 2012). Therefore, we expected an incremental use of the water surface during the acclimation period (just after the zebrafish are introduced in the novel experimental tank), with its maximum happening where food is added at the surface, followed by a drastic reduction after the approach of a simulated predator from the top (Figure S2; Video S1).

To capture the quantitative development of the behaviour of the shoal throughout the experimental assay, we subdivided the observations in 30-s periods. The bird-like simulated predator was released with a cable from a height of 1 m (Figure S2; Video S1) and was maintained above the tank for 5 s. By repeating the experiment 10 days later we estimated the repeatability of risk-related group behaviour.

### 2.3 | Circadian experimental set up and design

To test the absence of early peaks of locomotor activity associated with a decrease of risk-taking behaviour in the large size-selected line (according to our second hypothesis H2 in Figure 1), we recorded daily activity rhythms in a parallel experiment with a new set of adult zebrafish when they were at about 170 dpf. We randomly selected three groups of 15 fish for each of the six selection lines (six groups and 90 fish for each treatment: large-harvested, small-harvested and control line). The individual mass of the experimental fish groups was within the following range: large-harvested (3.6–5.7 g); control (3.3–6.5 g); small-harvested (4.6–6.1 g). The experimental setup was designed to jointly record swimming (as a proxy of locomotor activity) and self-feeding activity by a group of zebrafish (Figure S3; see also del Pozo et al., 2011).

Before starting experimental trials, fish were acclimated to laboratory conditions and to the use of the self-feeder for 15 days. Afterwards we recorded the swimming and self-feeding activity for 38 days (from at about 170 to 210 dpf) following established protocols in chronobiology to test for daily and circadian rhythmicity of fish behaviour (e.g. Sánchez-Vázquez & Tabata, 1998). The experimental trial was subdivided into four consecutive phases: (a) 12:12 hr LD cycle to investigate daily swimming and self-feeding rhythms (first LD trial: 15 days); (b) constant dim light (LL, 3.5 lux at the water surface) to investigate the endogenous free-running rhythms of swimming and self-feeding activity (constant conditions: 7 days); (c) 12:12 hr LD cycle to investigate their resynchronization (second LD trial: 15 days); (d) LL to ascertain the endogenous expression patterns of clock genes (final constant conditions: 2 days).

### 2.4 | Gene expression analysis

We measured gene expression at the end of the activity rhythms to test whether a decrease of risk-taking behaviour was associated

with the loss of circadian rhythmicity according to our third hypothesis (H3 in Figure 1). We selected seven genes controlling the most important functioning mechanisms of the circadian system in fish (Foulkes et al., 2016; Frøland Steindal & Whitmore, 2019; Idda et al., 2012; Vatine et al., 2011). The first group of genes was composed by genes related to the core transcriptional-translational feedback loop driving circadian oscillation in vertebrates (*per1b*, *clock1a*, *arntl1a*). The second group involved light-inducible genes (*per2*, *cry1a*). The third group was composed of genes related to the circadian clock output (*dbpa*, *tef1*). These three groups of genes were measured both in the brain and liver. Finally, a fourth group was composed of circadian clock-controlled genes (*lepa*, and *igf1*) related to growth and energy balance. *lepa* was measured both in brain and liver and *igf1* only in the liver. The product of the gene *lepa* acts as satiation signal in teleosts and has implications in energy balance and glucose homeostasis, it is mainly secreted in the liver with a daily rhythm of expression (Paredes et al., 2015; Rønnestad et al., 2017). The gene *lepa* is also expressed in the teleost brain, where expression increases after a meal (Yuan et al., 2016). The gene *igf1* is involved in growth processes and signals lipid metabolism and it is secreted with a daily rhythm of expression (Paredes et al., 2015).

Samples were collected throughout a 24-hr cycle at six different time points (Time of the day: 01:00, 05:00, 09:00, 13:00, 17:00, 21:00). Fish were exposed for 24 hr to the second constant dim light phase. We sampled a total of five fish for each time point and each treatment. Zebrafish were euthanized on ice and then the whole brain and liver were dissected and put in RNAlater at  $-80^{\circ}\text{C}$ . Total RNA was isolated using a Trizol reagent (Invitrogen) following the manufacturer's instructions. The amount, quality and composition of isolated RNA were analysed using Nanodrop ND-1000 (Thermo Fisher Scientific Inc.). DNase-treated RNA was used to perform cDNA synthesis in a final volume of 20  $\mu\text{l}$  using the iScript cDNA synthesis kit (Biorad). The reaction was performed at  $46^{\circ}\text{C}$  for 20 min, followed by a 1-min inactivation step at  $95^{\circ}\text{C}$ .

cDNA was PCR-amplified with the StepOnePlus Real-Time PCR System (Applied Biosystems) using SsoAdvanced™ Universal SYBR® Green Supermix (Biorad). The thermal cycling conditions were as follows: 30 s of denaturation at  $95^{\circ}\text{C}$ , followed by 40 cycles of a 15-s denaturation step at  $95^{\circ}\text{C}$  and then by an annealing-elongation step for 30 s at  $60^{\circ}\text{C}$ . After amplification, a melting curve analysis was performed to confirm amplicon specificity. The samples were run in triplicate. Gene-specific primers are indicated in Table S1. The relative expression levels of each sample were calculated by the  $2^{-\Delta\Delta\text{CT}}$  method (Livak & Schmittgen, 2001), using geometric mean of three housekeeping genes ( *$\beta$ actin*, *GADPH* and *EF1 $\alpha$* ) in both tissues.

### 2.5 | Statistical analysis

Risk-taking behavioural data were square root transformed and split into two sections: (a) before the approach of the simulated predator; (b) after the approach of the simulated predator. To assess differences



among the selection lines in the development of risk-taking behaviour of the shoals throughout the experimental assay, we modelled the data using non-orthogonal quadratic polynomial mixed models with selection lines as fixed factor (three levels) and consecutive 30-s time bins as covariate. Moreover, to assess differences among selection lines in risk-taking behaviour at specific time points of the experimental assay, we estimated a linear mixed model for each 30-s time bins with selection lines as fixed factor (three levels). In all cases, the different trials and replicates of the selection lines were used as random intercepts. Moreover, the square root transformed data were used to calculate adjusted repeatability (i.e. after controlling for fixed effects of selection lines; Nakagawa & Schielzeth, 2010) of risk-taking behaviour for each 30-s time bin across time (230–240 dpf). We used the same mixed effects models implemented before with selection lines as fixed factor (three levels) and replicates of the selection lines as additional random intercepts.

Waveform analysis (24-hr based) was carried out in both LD trials, and raw time series values (i.e. number of infrared light beam interruptions) were transformed in % of maximum to standardize the data. The Midline Estimating Statistic Of Rhythm (MESOR) was computed and represented as a horizontal threshold in waveform plots (Refinetti, 2006).

Regarding swimming activity rhythms, we estimated total activity for each group by calculating the area under the waveform curve during both LD trials (Refinetti, 2006). Next, early peaks of activity were calculated as the ratio between the percentages of area under the curve divided by the percentage of time of the period considered (i.e. 4 hr; Sbragaglia et al., 2013). Concerning self-feeding activity rhythms, we estimated feeding events for each group by calculating the area under the waveform curve during four different hourly sections (i.e. where most of the activity was concentrated): (a) from 06:00 to 07:00; (b) from 07:00 to 08:00; (c) from 08:00 to 09:00 (i.e. the hour before lights on); (d) from 09:00 to 10:00 (i.e. the hour after lights on). After a power transformation of the data, adjusted repeatability (selection lines as fixed effect and lines replicates as additional random effect into the model) were calculated over the two LD trials. Next, we modelled the data using the same mixed-effects model structure with selection lines as fixed effect and line replicates and LD trials as random intercepts.

Finally, circadian periodicity in gene expression was assessed using RAIN, a robust non-parametric method for the detection of rhythms in biological data that can detect arbitrary waveforms and has been widely applied in the measurement of circadian transcripts abundance (Thaben & Westermark, 2014).

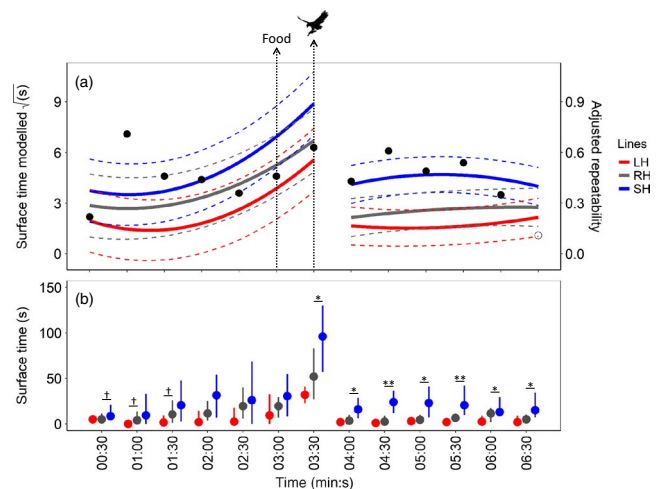
Periodogram and waveform analysis were performed using the software Eltemps ([www.el-temps.com](http://www.el-temps.com)). The rest of analyses were implemented using R 3.5.0 (<https://www.R-project.org/>) and the following additional packages; RAIN (Thaben & Westermark, 2014); RCOMPANION package for power transformation (<https://CRAN.R-project.org/package=rcompanion>), rptR for calculating repeatability and uncertainty via parametric bootstrapping (Stoffel et al., 2017), lme4 R package (Bates et al., 2014) to implement the linear mixed models, MuMIn to get marginal and conditional  $R^2$  (Bartoń, 2014). We used a 95% confidence interval.

## 3 | RESULTS

### 3.1 | Group risk-taking behaviour

Group risk-taking behaviour was overall significantly repeatable (adjusted  $R$  ranged from 0.22 to 0.71; Table S2)—after accounting for the fixed effects of selection lines —across time (230 and 240 dpf) with some differences depending on the 30-s time period considered (Table S2; Figure 2a; see text S2). We found a decrease of the adjusted repeatability towards the end of the experimental assay (Figure 2a; see Figure S4 for variance partitioning).

Group risk-taking behaviour before the approach of the simulated predator followed a positive quadratic trajectory, which was similarly expressed in both size-selected lines compared to the control (Table S3; Figure 2a). Zebrafish initially avoided the surface after being introduced into the experimental tank, but soon after started



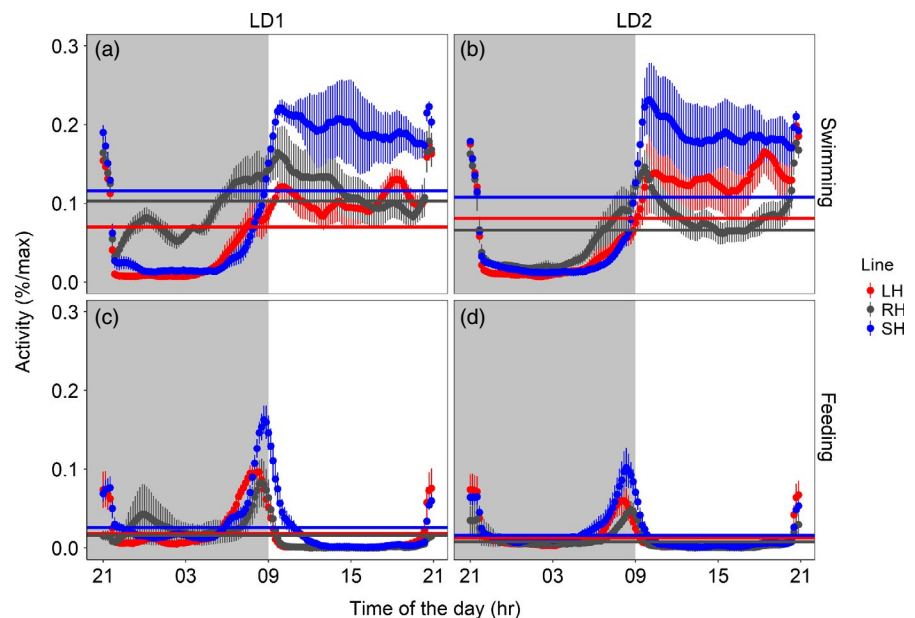
**FIGURE 2** Results of the diving test used to assess group risk-taking behaviour in the zebrafish selection lines in two consecutive trials at 230 and 240 dpf. Data are presented as cumulative time (bin in 30-s period) spent by zebrafish shoals at the surface of the water (7 cm; see Figure S2; Video S1) once moved to a novel tank. Food has been added at 03:00 and the predator approached at 03:30. We first model the data with a non-orthogonal quadratic polynomial mixed model by splitting the data into two sections: (i) before the approach of the simulated predator; (ii) after the approach of the simulated predator (a). Moreover, to assess differences among selection lines in risk-taking behaviour as specific time points of the experimental assay, we implemented a linear mixed model for each 30-s time bins (b). Finally, we calculated adjusted repeatability (i.e. after controlling for fixed effects of selection lines) of risk-taking behaviour for each 30-s time bin across time (230–240 dpf) by using the same mixed effects models implemented before (a). Repeatability scores (right axis in a) are presented as empty circles (when they were not significantly different from zero) and solid circles (when they were significantly different from zero; see also Tables S2–S4 and Figure S4 for further details and partitioning of variance components). The different colours represent the selection lines ( $N = 12$  for each selection line) together with confidence intervals (dashed coloured lines in a), median (points in b) and lower and upper quartiles (vertical lines in b). Bird pic source: <https://www.piqsels.com>. Significant difference is indicated by black horizontal lines ( $^{\dagger}p < 0.10$ ;  $^*p < 0.05$ ;  $^{**}p < 0.01$ )

to increase the time spent at the surface reaching the maximum when the food was added (03:30 in Figure 2). After the approach of the simulated predator (04:00 in Figure 2), all the selection lines reduced their group risk-taking behaviour (Figure 2). The small-harvested line spent significantly more time at the surface, i.e. individuals took more risk during feeding (+6.5 s) and after the approach of the simulated predator (ranging from +2.0 to +5.7 s; Figure 2b; Table S4), while the large-harvested line only showed an almost significant effect in being less risk-taking than the control during the habituation phase (−3.2 and −3.4 s; Figure 2b; Table S4).

**TABLE 1** The output of the periodogram analysis for swimming and self-feeding activity rhythms during the three steps of the experiment (first light-dark trial: LD1; constant dim light: LL1; and second light-dark trial: LD2) according to the three selection lines (large-harvested: LH, small-harvested: SH and control: RH). Periods (*T*) or free running periods (*tau*) are averaged according to the number of groups (*N*; the proportion indicates the number of groups with significant rhythms) and the robustness of periodicities is expressed as percentage of variance explained (%*V*) together with the midline estimating statistic of rhythm (MESOR). The standard errors are reported between parentheses

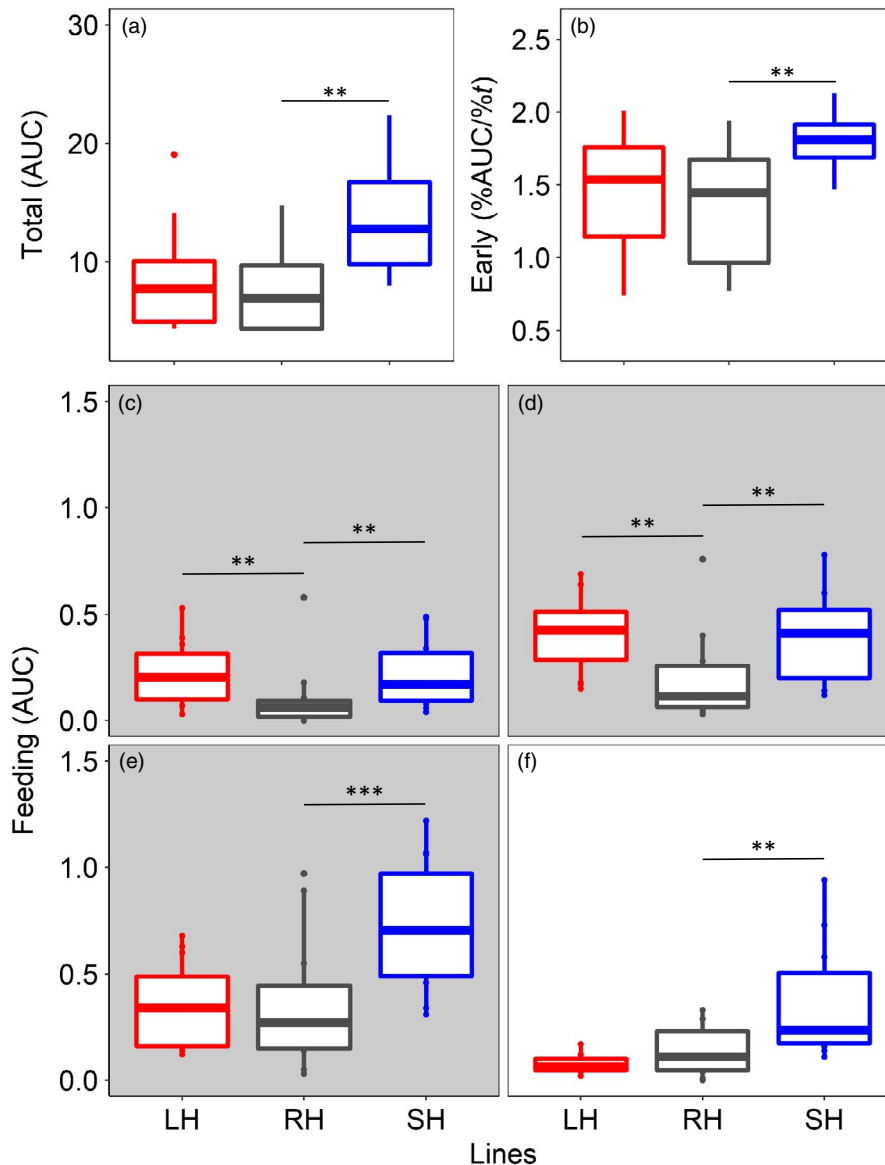
Phenotype	Experimental phase	Line	<i>N</i>	<i>T</i> or <i>tau</i> (SE)	% <i>V</i> (SE)	MESOR (SE)
Swimming	LD1	LH	6/6	24.00 (0.00)	47.41 (4.54)	0.070 (0.006)
		RH	6/6	24.00 (0.02)	29.74 (10.38)	0.103 (0.011)
		SH	5/6	24.02 (0.02)	52.48 (10.13)	0.116 (0.012)
	LL1	LH	4/6	20.88 (1.70)	29.43 (11.23)	—
		RH	2/6	26.33 (0.00)	30.29 (8.59)	—
		SH	2/6	23.42 (0.33)	23.54 (1.89)	—
	LD2	LH	6/6	24.03 (0.02)	52.45 (8.54)	0.081 (0.017)
		RH	6/6	24.00 (0.03)	51.01 (14.30)	0.066 (0.006)
		SH	5/6	24.00 (0.00)	56.96 (20.82)	0.108 (0.018)
Self-feeding	LD1	LH	6/6	24.00 (0.00)	46.44 (7.45)	0.018 (0.003)
		RH	6/6	23.97 (0.02)	38.89 (18.18)	0.016 (0.009)
		SH	6/6	24.01 (0.03)	43.21 (6.35)	0.026 (0.005)
	LL1	LH	4/6	23.56 (0.52)	28.59 (6.73)	—
		RH	3/6	22.56 (1.31)	18.86 (3.43)	—
		SH	1/6	16.83 (—)	16.03 (—)	—
	LD2	LH	6/6	24.03 (0.02)	46.07 (11.67)	0.013 (0.003)
		RH	6/6	24.01 (0.01)	32.29 (7.92)	0.008 (0.003)
		SH	6/6	24.00 (0.02)	37.87 (10.17)	0.016 (0.003)

**FIGURE 3** Mean waveforms (time scale 24 hr) for swimming (a, b) and self-feeding (c, d) activity of zebrafish shoals are expressed as percentage of the maximum during the first (LD1: days 1–15) and second (LD2: days 22–36) light-dark trial. Each point represents the 10-min binned mean across all the experimental days in LD1 and LD2 for all the groups. The different colours represent the selection lines: red for large-harvested (LH; *N* = 6) grey for control (RH; *N* = 6) and blue for small-harvested (SH; *N* = 5). The horizontal lines represent the midline estimating statistic of rhythm as reported in Table 1. The vertical lines represent the standard error (*N* between 75 and 90). Grey shadowed areas represent the dark hours (lights on is at time of the day = 09:00)



### 3.2 | Behavioural activity rhythms

The behavioural rhythms of all the selection lines revealed significant 24-hr periodicities during both LD trials (first LD trial, days 1–15; second LD trial, days 22–36) and significant free-running rhythmicity under LL conditions, with periods close to 24-hr (Figure S5; Table 1, see text S3). All lines displayed typical diurnal swimming activity rhythms with greater activity during photophase (Figure 3a,b). However, we detected fine-scale timing differences in several daily behavioural rhythms among the lines (Figure 4).



**FIGURE 4** Total swimming activity during light hours (a; area under the waveform curve, AUC) and early daily activity (b; percentage of activity during the first 4 hr of light) together with self-feeding activity (c–f; area under the waveform curve, AUC) during the last 3 hr of scotophase (grey shadow; c = 06:00–07:00; d = 07:00–08:00; e = 08:00–09:00) and the first hour of photophase (f = 09:00–10:00). The different colours represent the selection lines: red for large-harvested (LH) grey for control (RH) and blue for small-harvested (SH); *N* between 5 and 6 (see Table 1 for more details). Boxplots represent the median (bold centreline), the 25th (top of the box) and 75th percentile (bottom of the box). Significant differences are indicated by black horizontal lines (\*\**p* < 0.01; \*\*\**p* < 0.001; see text for more details)

Specifically, total swimming activity during the photophase was significantly ( $p < 0.001$ ) repeatable—after accounting for the fixed effects of selection lines—over the two LD trials ( $R = 0.78$  [CI: 0.27–0.92]; Figure S6a). The small-harvested line was significantly ( $t_8 = 3.62$ ;  $p < 0.01$ ) more active than controls (Figure 4a), while no significant differences ( $t_8 = 0.73$ ;  $p = 0.473$ ) were detected between the large-harvested line and controls (Figure 4a). Early peaks of swimming activity were also significantly ( $p < 0.05$ ) repeatable ( $R = 0.46$  [CI: 0.070–0.80], after accounting for the fixed effects of selection lines; Figure S6b). In particular, the small-harvested line concentrated significantly ( $t_8 = 3.26$ ;  $p < 0.01$ ) more daily activity on the first 4 hr of the photophase relative to controls (Figure 4b), while no significant differences ( $t_8 = 0.64$ ;  $p = 0.524$ ) were detected between the large-harvested line and controls (Figure 4b).

The lines displayed self-feeding activity rhythms during the last hours of darkness (Figure 3c,d). The self-feeding activity was significantly ( $p < 0.05$ ) repeatable (Figure S6c–f) over the two LD trials in the four consecutive hourly sections that were analysed (from 3 hr before to 1 hr after light-on, Figure S6c–f). At the onset of the self-feeding events

(06:00–07:00 hr; darkness) both size-selected lines were significantly (large-harvested line:  $t_8 = 2.94$ ,  $p < 0.01$ ; small-harvested line:  $t_{22} = 2.85$ ,  $p < 0.01$ ) more active than controls (Figure 4c). The same pattern (large-harvested line:  $t_8 = 3.37$ ,  $p < 0.01$ ; small-harvested line:  $t_8 = 3.10$ ,  $p < 0.01$ ) was detected in the next hourly section (07:00–08:00 hr; darkness, Figure 4d). Subsequently, during the hour before light-on (08:00–09:00 hr), the small-harvested line was significantly ( $t_8 = 3.64$ ,  $p < 0.001$ ) more active in demanding food than controls (Figure 4e), while the large-harvested line did not show significant differences relative to the control ( $t_8 = 0.61$ ,  $p = 0.545$ ). The same pattern (small-harvested line:  $t_8 = 3.49$ ,  $p < 0.01$ ; large-harvested line:  $t_8 = -0.43$ ,  $p = 0.672$ ) was detected during the first hour after light-on (09:00–10:00 hr; Figure 4f).

### 3.3 | Core clock genes

Core clock genes expression revealed significant differences in their circadian oscillations among the zebrafish lines (Table 2).



**TABLE 2** The output of the analysis with RAIN (a robust non-parametric method for the detection of rhythms in biological data) regarding the circadian oscillation of transcript abundance of the genes related to the circadian system and to neuroendocrinological aspects of energy balance, appetite and growth. Gene expression was measured at the end of the experiment in constant dim light. Transcripts are subdivided according to their role for each selection lines (large-harvested: LH, small-harvested: SH and control: RH) and in each tissue (brain and liver). The timing of the peak of the circadian oscillation is indicated using the time of the day in hours (TD; see also Figure 5) together with the *p* value and total *N* (*N* is between 4 and 5 for each time point and selection line). Significant circadian rhythmicity is reported in bold

Clock mechanism	Gene	Line	Brain			Liver		
			<i>N</i>	Peak	<i>p</i> value	<i>N</i>	Peak	<i>p</i> value
Core clock	<i>arntl1a</i>	LH	<b>29</b>	<b>01</b>	<b>&lt;0.01</b>	30	01	0.086
		RH	26	09	0.052	<b>30</b>	<b>09</b>	<b>&lt;0.001</b>
		SH	30	01	0.682	<b>30</b>	<b>09</b>	<b>&lt;0.01</b>
	<i>clock1a</i>	LH	<b>28</b>	<b>01</b>	<b>&lt;0.01</b>	<b>28</b>	<b>09</b>	<b>&lt;0.001</b>
		RH	<b>28</b>	<b>09</b>	<b>&lt;0.05</b>	30	09	0.181
		SH	30	05	0.279	<b>30</b>	<b>09</b>	<b>&lt;0.001</b>
	<i>per1b</i>	LH	<b>29</b>	<b>21</b>	<b>&lt;0.001</b>	<b>29</b>	<b>01</b>	<b>&lt;0.01</b>
		RH	<b>29</b>	<b>17</b>	<b>&lt;0.01</b>	30	17	0.751
		SH	<b>29</b>	<b>21</b>	<b>&lt;0.001</b>	<b>29</b>	<b>21</b>	<b>&lt;0.05</b>
Light-inducible	<i>per2</i>	LH	<b>30</b>	<b>21</b>	<b>&lt;0.001</b>	30	01	0.376
		RH	27	01	0.117	30	01	0.054
		SH	<b>28</b>	<b>17</b>	<b>&lt;0.05</b>	30	05	0.308
	<i>cry1a</i>	LH	<b>30</b>	<b>21</b>	<b>&lt;0.001</b>	<b>28</b>	<b>21</b>	<b>&lt;0.01</b>
		RH	<b>30</b>	<b>17</b>	<b>&lt;0.001</b>	<b>30</b>	<b>01</b>	<b>&lt;0.01</b>
		SH	<b>30</b>	<b>01</b>	<b>&lt;0.001</b>	30	05	0.120
Clock output	<i>tef1</i>	LH	<b>29</b>	<b>17</b>	<b>&lt;0.05</b>	<b>30</b>	<b>17</b>	<b>&lt;0.05</b>
		RH	<b>30</b>	<b>17</b>	<b>&lt;0.001</b>	<b>30</b>	<b>21</b>	<b>&lt;0.001</b>
		SH	<b>29</b>	<b>17</b>	<b>&lt;0.05</b>	<b>30</b>	<b>17</b>	<b>&lt;0.05</b>
	<i>dbpa</i>	LH	29	17	0.078	30	17	0.144
		RH	<b>30</b>	<b>17</b>	<b>&lt;0.05</b>	29	17	0.205
		SH	<b>29</b>	<b>17</b>	<b>&lt;0.001</b>	28	17	0.190
Appetite Food intake	<i>lepa</i>	LH	<b>27</b>	<b>17</b>	<b>&lt;0.05</b>	27	05	0.769
		RH	30	17	0.456	28	17	0.319
		SH	<b>30</b>	<b>17</b>	<b>&lt;0.01</b>	30	17	0.876
Growth	<i>igf1</i>	LH	—	—	—	28	13	0.248
		RH	—	—	—	<b>28</b>	<b>13</b>	<b>&lt;0.001</b>
		SH	—	—	—	30	13	0.134

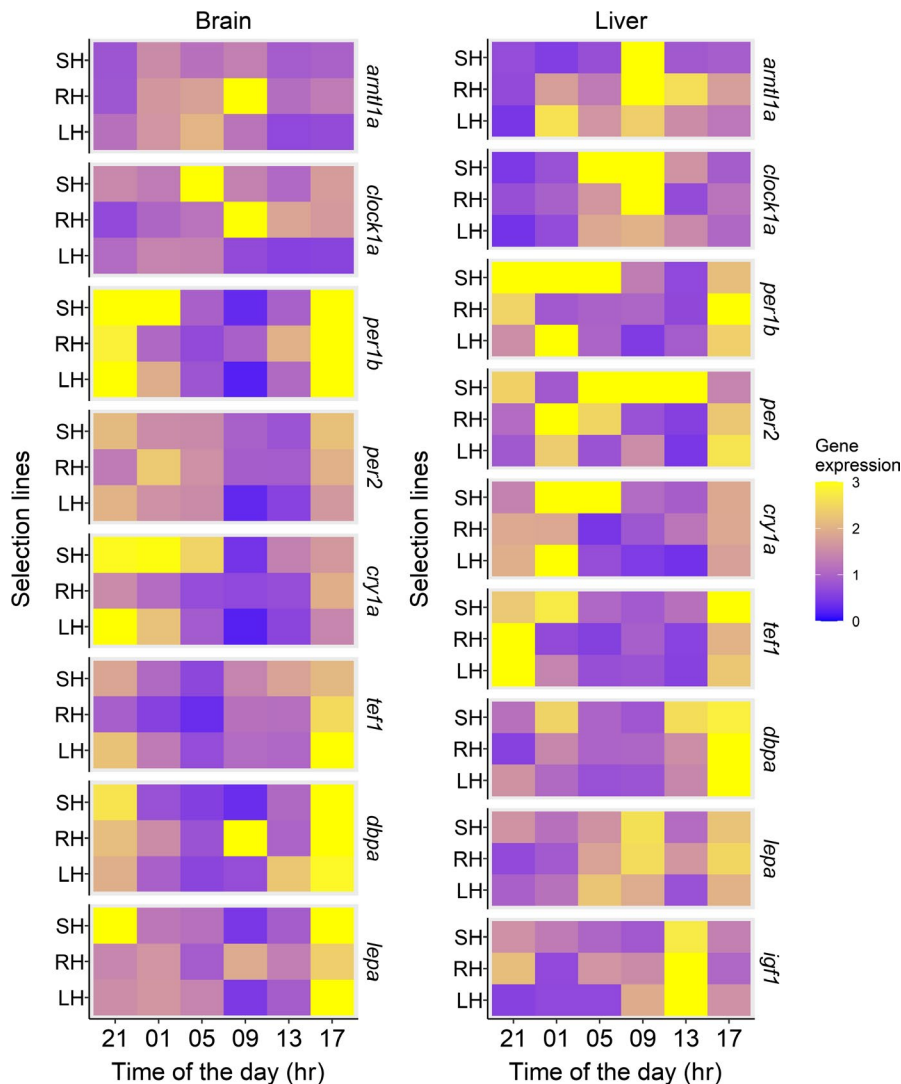
In the brain, the control line showed an almost significant effect ( $p = 0.052$ ) in the circadian rhythmicity of *arntl1a* and a clear circadian oscillation of *clock1a* with a peak at the same time of the day (TD) of *arntl1a* (TD09 for both genes; Table 2). By contrast, the small-harvested line did not reveal circadian rhythmicity of *arntl1a* and *clock1a* compared to the control, while the large-harvested line shifted its peaks of circadian expression for both genes by 8 hr (TD01 instead of TD09, Table 2; Figure 5). Results of *per1b* indicated significant circadian patterns in the three lines with main differences in peaks in both size-selected lines that shifted from TD17 to TD21 relative to the control (Figure 5; Table 2). In the liver, the small-harvested line showed similar circadian rhythmicity to the control line in terms of *arntl1a*, while the large-harvested line revealed an almost significant effect ( $p = 0.086$ ) in the circadian oscillation (Table 2). The control line did not show any circadian rhythmicity in terms of *clock1a* and *per1b*, however, the size-selected lines revealed clear circadian rhythmicity for these two genes with slightly different peaks for *per1b* (Table 2; Figure 5).

### 3.4 | Light-inducible genes

In the brain, the control line showed no significant rhythmicity of *per2*, while the size-selected lines oscillated with peaks at different times (TD21 and TD17 for large- and small-harvested lines respectively; Table 2; Figure 5). Results of *cry1a* indicated significant circadian rhythmicity with different peaks in the size-selected lines relative to the control: the large-harvested line shifted the peak from TD17 to TD21, while the small-harvested line shifted from TD17 to TD01 (Table 2; Figure 5). In the liver, both size-selected lines lost circadian rhythmicity of *per2* with respect to the control that showed an almost significant effect ( $p = 0.054$ ). The small-harvested line lost rhythmicity for *cry1a*, while the peak of the large-harvested line shifted from TD01 to TD21 compared to the control (Table 2; Figure 5).

### 3.5 | Clock output genes

The circadian expression of clock output genes showed few peak shifts compared to the other two groups of genes that we



**FIGURE 5** Mean endogenous (i.e. measured under constant dim light) transcript abundance of the genes related to the circadian system and clock-controlled genes related to growth and energy balance in the brain (left) and liver (right) at different times of the day for the three selection lines (large-harvested: LH, small-harvested: SH and control: RH;  $N$  between 4 and 5, see Table 2 for  $p$  values and more details)

investigated (Table 2). In the brain, the three lines had a circadian rhythmicity of *tef1* and *dbpa* with peaks at the same time (TD17) except for *dbpa* in the large-harvested line, which only showed an almost significant effect ( $p = 0.078$ ; Table 2; Figure 5). In the liver, both size-selected lines shifted the peak of the circadian oscillation of *tef1* from TD21 to TD17 compared to the control (Table 2; Figure 5). By contrast, the three lines lost circadian rhythmicity in terms of *dbpa* (Table 2; Figure 5).

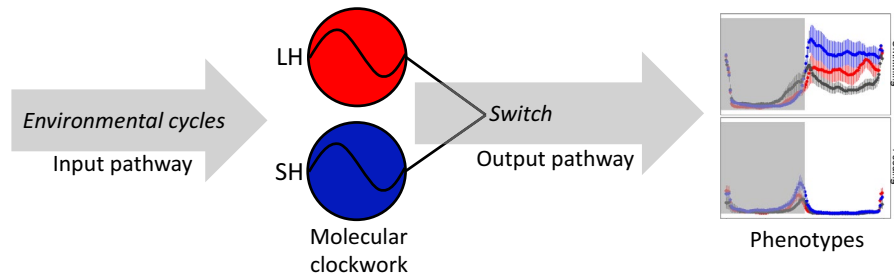
### 3.6 | Clock-controlled genes related to growth and energy balance

The circadian expression of *lepa* (involved in metabolism and energy balance) and *igf1* (involved in growth) also revealed significant differences between the size-selected lines and controls. The circadian oscillation of *lepa* expression in the brain was only significant in the size-selected lines, but not in the control, with peaks at the same time (TD17; Table 2; Figure 5). By contrast, we did not find significant circadian oscillation of *lepa* in the liver (Table 2; Figure 5).

Finally, the *igf1* circadian oscillation in the liver was significant only in the control line (Table 2; Figure 5).

## 4 | DISCUSSION

We found that five generations of size-selective harvesting left an evolutionary legacy in group risk-taking behaviour as well as in the circadian behavioural and molecular outputs. Three key results are noteworthy and further discussed below. First, size-selective harvesting fostered evolutionary changes of group risk-taking behaviour, which were more evident in the small-harvested line than large-harvested line compared to the control. Second, we found changes in fine-scale daily behavioural rhythms in the small-harvested line, but not in large-harvested line, compared to the control. Specifically, the small-harvested line was overall more risk-taking, more active during photophase, and concentrated more activity early in the photophase compared to the control. Moreover, the small-harvested line extended self-feeding activity to the photophase where it showed a higher self-feeding activity than the



**FIGURE 6** The conceptual framework of the downstream switch we proposed to explain the results presented here. The circadian clock synchronized with the environmental cycles (e.g. light–dark cycle or feeding). Next, the size-selective harvesting (large-harvested in red: LH; small-harvested in blue: SH) triggers the evolution of different molecular circadian clockwork with respect to control. Finally, in the output pathway the different molecular circadian clockwork is switched to produce a similar output driving the overall daily activity rhythms phenotypes (i.e. swimming during light hours and self-feeding activity in the last hours of darkness)

control. All together, these results suggest asymmetric evolutionary changes induced by size-selective harvest in relation to group risk-taking behaviour and fine-scale timing of swimming and self-feeding daily activity rhythms. Third, from a molecular perspective, our data suggest the presence of a switch in the circadian output pathway that buffered the molecular circadian clockwork (Figure 6). In fact, we found changes in the molecular circadian clockwork of both size-selected lines. These changes were expected to affect overall temporal patterns of swimming and self-feeding activity. However, all lines showed overall similar temporal patterns of diurnal swimming and self-feeding in the last hours of darkness and they only differed significantly in the fine-scale timing of these behaviours. We suggest being overall active during light hours and looking for food during the last hours of darkness may have important adaptive value for zebrafish, no matter what the molecular clock is signalling. Therefore, our results indicate that size-selective mortality induces changes in the molecular clock and in the fine-scale timing of daily behavioural rhythms, but overall timing (diurnal swimming and self-feeding in the last hours of darkness) revealed evolutionary resistance (*sensu* Sgrò et al., 2011), which buffered the changes in the molecular clockwork.

#### 4.1 | Group risk-taking behaviour

We found group risk-taking behaviour to be a repeatable behaviour and thus indicative of a zebrafish collective personality trait (Bengston & Jandt, 2014; Jolles et al., 2018). We also documented that consistency of group behaviour can vary during an experimental assay as demonstrated before for individual traits (e.g. O'Neill et al., 2018). We found partial support for our first hypothesis (H1) in terms of the small-harvested line significantly increasing group risk-taking behaviour compared to the control during feeding and after the simulated predator approach; while the large-harvested line almost significantly decreased group risk-taking behaviour compared to the control during the habituation phase. The match/mismatch between the species-specific evolutionary history and human-induced rapid environmental changes may be key to understand behavioural responses, such those observed in our experimental system (Sih et al., 2011). The surface of the water is a risky environment for

zebrafish (Cachat et al., 2011; Spence et al., 2008), therefore it is expected that the time spent feeding at the surface is under predation-driven selection pressure in wild populations and that genetic variation for surface feeding is present in the wild (Sih et al., 2011). We started our experimental system with wild zebrafish populations and fed them with clumped food at the surface (Uusi-Heikkilä et al., 2015); therefore the time spent feeding at the surface played a major role in determining size-at-harvest. Our selection pressure was divergent, either favouring large or small size-at-harvest. However, we did not observe symmetrical evolutionary changes in the two opposing selection lines in terms of group risk-taking behaviour, at least not when judged based on a strict measure of statistical significance (e.g.,  $p < 0.05$ ). Our hypothesis was fully confirmed for the small-harvested line, which took more risk during foraging and after the approach of the predator and we suggest the behavioural changes in terms of group risk-taking behaviour followed the selection on size-at-harvest through a correlated selection response. Such interpretation would imply that the evolutionary changes of risk-taking behaviour in the small-harvested line were driven by an energy acquisition pathway (Enberg et al., 2012), coupling behaviour and size-at-harvest such that selection on size-at-harvest also altered risk-taking behaviour (Biro & Post, 2008). By contrast, the evolutionary changes of risk-taking behaviour in the large-harvested line were only present as an almost significant effect and exclusively in the habituation phase. The initially expected adaptive changes towards a decrease in boldness could have been buffered by an antagonistic effect of an energy acquisition mechanism (i.e. where the expected trend of evolution is indeed a decrease of boldness), and a counter response caused by the evolution of a fast life history (i.e. where the expected trend of evolution is increased boldness). Similarly, in a theoretical model Andersen et al. (2018) predict that large size-selective harvesting could either foster bold or shy fish, depending on the strength of size-selection and correlations of behaviour and growth rate. These possible counterforces caused by life history adaptations could explain the weak response in terms of group risk-taking behaviour of the large-harvested line. Indeed, life history adaptations were more evident in the large-harvested line than the small-harvested line with respect to control (Uusi-Heikkilä et al., 2015), and thus may be expected to have had a stronger evolutionary effect on

risk-taking behaviour than an energy acquisition pathway. In summary, size-selective mortality did not affect the overall trajectory in group risk-taking behaviour during the experimental assay; however, it triggered asymmetrical evolutionary changes both in terms of strength and when specific differences were observed (habituation phase, feeding or after the simulated predator approach).

Previous work in our laboratory using the same experimental system was conducted at the individual level, and results related to individual risk-taking behaviour in response to size-selection showed different patterns than those reported here at the group level. Specifically, Uusi-Heikkilä et al. (2015) reported that juveniles of the small-harvested line increased individual risk-taking compared to controls, while Sbragaglia, Alós, et al. (2019) showed that adult females decreased risk-taking behaviour relative to controls. Similarly, the large-harvested line did not show changes in individual risk-taking behaviour compared to controls in both juveniles (Uusi-Heikkilä et al., 2015) and adults (Sbragaglia, Alós, et al., 2019).

Two main limitations of the previous experimental approaches that were overcome in this research may explain some of the discrepancies. The first is related to the behavioural assay. Our previous work focused on individual behavioural traits using the open field test to assess risk-taking behaviour (Sbragaglia, Alós, et al., 2019; Uusi-Heikkilä et al., 2015). An open field test is used to measure explorative behaviour, rather than risk-taking behaviour (Réale et al., 2010). Moreover, it disregards the third vertical dimension, which constitutes an important behavioural aspect for zebrafish both due to its evolutionary history and because of the selection environment experienced in our experimental system where fish were held in large tanks and food was provided on the surface. Thus, the experimental approach used in the present paper seems more appropriate than the open field to reveal evolutionary changes in risk-taking behaviour of the zebrafish size-selected lines. The second limitation is that our previous behavioural assays focused on isolated individuals instead of examining the behavioural phenotype of groups (Sbragaglia, Alós, et al., 2019). Being in a group has important consequences for foraging behaviour in zebrafish (Harpaz & Schneidman, 2019; Pitcher, 1986), and social isolation can create stress-related behaviours (Shams et al., 2017). We propose that zebrafish expressed a more reliable behaviour in the group context presented here than in the open field experiments previously reported on isolated individuals. Collectively, the evolutionary changes of risk-taking presented in this paper seem more robust and they suggest that testing evolution of behaviour in response to experimental size-selective harvest should carefully consider the match between evolutionary history of the species, the environmental conditions experienced during the artificial selection and the behavioural assay used to reveal the evolutionary basis of behavioural changes (Klefoth et al., 2012).

## 4.2 | Daily behavioural activity rhythms

We found partial support for our second hypothesis (H2) because the small-harvested line was found to be more active during the

photophase and concentrated its swimming activity at the beginning of the scotophase. However, we did not find significant changes in the large-harvested line compared to controls. The changes in daily activity rhythms of the small-harvested line agree with a study demonstrating that proactive zebrafish (i.e. risk-taking individuals) are more active in the first hours of photophase compared to reactive ones (Tudorache et al., 2018). Moreover, the self-feeding activity events of the small-harvested line extended to the hours before and after light-on. The timing of feeding is a key aspect for survival and is traded-off against predation risk and food availability (Kronfeld-Schor & Dayan, 2003). The ecological significance of zebrafish feeding during the last hours of darkness may be related to avoidance of visual predators (e.g. birds; Spence et al., 2008) and/or to storing energy to be prepared for spawning that occurs at the beginning of photophase (del Pozo et al., 2011). However, the food demand sensor was located close to the water surface (del Pozo et al., 2011; see also Figure S3) and an increase of self-feeding events towards light-on by the small-harvested line could also indicate additional support for the evolutionary change of group foraging in a risky context following a resource acquisition mechanism that fostered a correlated selection response of size-at-harvest and risk-taking behaviour.

Overall, the results related to fine-scale timing of daily behavioural rhythms revealed that the size-selected lines responded differently, indicating asymmetrical evolutionary changes in response to size-selection, as previously reported for life-history traits in other selection experiments with fish (Amaral & Johnston, 2012; Renneville et al., 2020; van Wijk et al., 2013). Harvesting-induced evolution of behaviour likely has a complex and multivariate nature (Claireaux et al., 2018; Renneville et al., 2020), thereby complicating generalizations related to evolutionary correlations among traits. The somewhat asymmetrical evolutionary changes in the two size-selected lines might be related to the complex interplay of evolutionary mechanisms such as life history adaptations and energy acquisition mechanisms as explained above. However, other mechanisms could also be at play such as genetic trade-offs or functional limitations in our model species (Arnold, 1992; Renneville et al., 2020).

## 4.3 | Circadian system switch

We rejected our third hypothesis (H3) because we did not find a decrease of the circadian clock gene rhythmicity (a decrease of robustness and amplitude as documented by Tudorache et al., 2018) linked to a decrease of group risk-taking behaviour. A possible explanation could be related to the fact that the two recent studies on zebrafish that demonstrated a relation between individual personality trait and the circadian system (i.e. proactivity/reactivity; Rey et al., 2013; Tudorache et al., 2018), measured transcript abundance under light-dark conditions. Thereby, the truly endogenous nature of the circadian molecular clockwork could have been masked by the light-dark cycle (i.e. direct response without synchronizing the circadian molecular clockwork; Dunlap et al., 2004;

Mrosovsky, 1999). In our study, we measured transcript oscillations under constant conditions, thereby providing a truly endogenous effect of size-selective harvesting on the molecular circadian clockwork.

All genes except *tef1* and *dbpa* had different peaks in the three lines, while in few cases the two size-selected lines peaked at the same time (e.g. *per1b* in the brain). Collectively, these results indicate differences in the circadian molecular clockwork of the size-selected lines, suggesting that size selection can alter the molecular basis of the circadian clock. However, the similar peaks recorded in the genes related to the clock output pathway (*tef1* and *dbpa*) implicated the occurrence of a switch (a plastic response in terms of distribution of activity through the time of the day; see review by Mrosovsky & Hattar, 2005) downstream to the circadian clock, especially in the brain. There are three mechanisms used to explain circadian system switches (Hut et al., 2012): (a) altered properties of the circadian clock leading to different activity patterns; (b) identical properties of the circadian clock switching in the output pathway, leading to different activity patterns; and (c) identical properties of the circadian clock subjected to masking that ultimately determine the activity patterns. However, none of them fit our results. We instead suggest the presence of a fourth mechanism where altered properties of the circadian clock are switched in the molecular output pathway, leading to overall similar timing of daily activity patterns at the behavioural level (in our case, overall diurnal swimming and self-feeding in the last hours of darkness, Figure 6). The role of the clock output in determining diurnal and nocturnal phenotypes is also supported by a recent study with the fruit fly *Drosophila melanogaster* (Pegoraro et al., 2018), where ten generations of artificial selection for diurnal and nocturnal phenotypes were sufficient to obtain highly divergent strains where most differentially expressed genes were associated with the clock output pathway.

The occurrence of a switch in the circadian system was also reinforced by the fact that the size-selected lines at  $F_9$  showed genetic changes in areas of genes associated with serotonin synthesis (Uusi-Heikkilä et al., 2015). Serotonin plays an important role in feeding and other behaviours in fish (Lillesaar, 2011; Paredes et al., 2015; Piccinetti et al., 2013), and it is also a key element in the synthesis of melatonin (Lima-Cabello et al., 2014), which represents a major rhythmic output of the fish circadian system controlling photoperiodic-dependent functions and synchronization of biological processes (Falcón et al., 2010; Lima-Cabello et al., 2014). Thus, the serotonergic system could have played a role in the switching mechanism we document in response to size-selective harvest (Uusi-Heikkilä et al., 2015).

#### 4.4 | Energy balance and growth

The *lepa* expression in the brain displayed significant circadian variations in both size-selected lines with a peak at the same circadian time compared with the absence of rhythmicity in the control group.

Moreover, both size-selected lines showed a disruption of the *igf1* circadian rhythm in the liver. Results on both genes suggest a modification of growth, lipid and protein metabolism and energy balance (Piccinetti et al., 2013). Modifications of the insulin growth factors pathways in the zebrafish's skeletal muscle have also been documented in previous size-selection experiments with zebrafish in other labs (Amaral & Johnston, 2012), supporting the idea that this pathway might have a major role in mediating growth processes in response to size-selection.

#### 4.5 | Limitations and translation of results

Although our common-garden approach controlled for much of possible non-genetic factors, subtle differences in the environment among the selection lines could have arisen from body size differences (see text S1), thereby creating differences in rearing biomass density or dominance hierarchies. These environmental effects could have also shaped the group behavioural phenotypes independently of a genetic-based legacy left by size-selective harvesting (e.g. Magnhagen, 2015). Additionally, selection was relaxed for eight generations and selection lines could have been also affected by genetic drift, but previous results on the same selection lines at  $F_{11}$  indicated that gene expression changes cannot be explained by genetic drift alone (Uusi-Heikkilä et al., 2017). These evidences support the idea of line-specific evolutionary responses. Another aspect to be considered is that we did not take into consideration inter-individual differences in group behaviour and gene expression and how they could affect the results presented in this paper. All these aspects are certainly interesting for future studies aiming at characterizing cause and effect of size-selective mortality on behavioural traits and their molecular control.

Importantly, the translation of our findings to real fisheries or natural predation contexts should be done with great care giving the experimental nature of our system. For example, in most eco-evolutionary contexts fish have multiple spawning events with overlapping generations and behaviour is probably under direct harvest selection as well (Arlinghaus et al., 2017). By contrast, in our experimental selection scenario the actual harvest decision was entirely based on size-at-harvest, and not by the behaviour of the zebrafish towards any fishing gear. Our work thus should be considered as experimental evidence that evolution of group risk-taking behaviour and the circadian system in response to size-selective mortality is generally plausible (either in response to human or non-human predators), motivating more research in the wild. Indeed, a recent study indicated that the genomic basis for growth rate divergence in response to experimental size-selective harvesting recapitulated responses to size-selection gradients seen in the wild (Therkildsen et al., 2019). Therefore, experimental systems such as the one used here carry basic scientific information that harvesting or other forms of mortality can have evolutionary effects that extend beyond environmental effects.



## 5 | CONCLUSIONS

The main impacts of human activities on biological rhythms of wild-life are related to diurnal disturbance as documented by an increase of nocturnality in mammalian species in response to human presence (Gaynor et al., 2018). Another important human-induced change of activity rhythms is represented by artificial light at night that has been demonstrated to have a profound impact on circadian rhythms in a wide range of taxa (Gaston et al., 2015). Our work adds to this literature by showing that size-selective mortality (simulating eco-evolutionary contexts typical of fisheries or natural predation) could evolutionarily change group risk-taking behaviour and the circadian system. Further research needs to clarify the possibly adaptive and maladaptive consequences of harvesting-induced evolutionary changes of group risk-taking behaviour (e.g., Sbragaglia, Klamser, et al., 2019), as well as the fitness costs of the molecular circadian switch proposed here.

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## AUTHORS' CONTRIBUTIONS

V.S., J.F.L.-O., C.B. and R.A. designed the research; V.S., J.F.L.-O. and E.F. performed the research; V.S. analysed the data; V.S., J.F.L.-O., E.F., C.B. and R.A. interpreted the results; V.S. and R.A. wrote the paper with contributions from all the co-authors.

## DATA AVAILABILITY STATEMENT

Data and the relative R script are available on Dryad Digital Repository <https://doi.org/10.5061/dryad.0v0t4b8pg> (Sbragaglia et al., 2020).

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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